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Characterization of a transport activity for long-chain peptides in barley mesophyll vacuoles

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Abstract

The plant vacuole is the largest compartment in a fully expanded plant cell. While only very limited metabolic activity can be observed within the vacuole, the majority of the hydrolytic activities, including proteolytic activities reside in this organelle. Since it is assumed that protein degradation by the proteasome results in the production of peptide with a size of 3 to 30 amino acids, we were interested whether the tonoplast exhibits a transport activity, which could deliver these peptides into the vacuole for final degradation. Here we show that isolated barley mesophyll vacuoles take up peptides with a size of 9 to 27 amino acids in a strictly ATP-dependent manner. Uptake is inhibited by vanadate but not by NH_4^+, while GTP could partially substitute for ATP. The apparent affinity for the 9-mer peptide was 15 µM, suggesting that peptides are efficiently transferred to the vacuole *in vivo*. Inhibition experiments showed that peptides with a chain length below 10 amino acids did not compete as efficiently as longer peptides for the uptake of a 9 amino acid long peptide. Our results suggest that vacuoles contain at least one peptide transporter that belongs to the ABC-type transporters, which efficiently exports long chain peptides from the cytosol into the vacuole for final degradation.

Introduction

Plant vacuoles are multifunctional organelles that play a central role during the whole plant life. While young cells often contain many small vacuoles, most of the mature cells have one central vacuole, which occupies up to 90% of the cell volume. Additionally, small vacuoles may be present in such cells. A large number of substances such as inorganic ions, soluble carbohydrates, organic acids, amino acids, secondary compounds or modified xenobiotics, but also hydrolytic and biosynthetic enzymes, can be found within vacuolar compartments. The vacuole is responsible for numerous processes, sometimes unique to the plant cell [Martinoia *et al.*, 2007, Hörtensteiner & Feller, 2002]. The negative water potential drives the uptake of water into the large central vacuole, generating the turgor pressure that enables the cells to expand, as well as to sustain the shape of non-lignified plants. The vacuole can serve as a transient storage compartment for nutrients, which can be released when required for growth and development. Furthermore, in specialized storage tissues or seeds, storage vacuole can act as storage compartment for protein. The vacuole plays also a crucial role in the tight control of cytosolic concentrations of metabolites and ions for cell homeostasis. Additionally, the vacuole is implicated in detoxification processes and defence responses by accumulating and sequestrating toxic compounds and defence molecules.

In order to support the intensive exchanges that occur between the cytosol and the vacuole a large number of proteins are embedded in the vacuolar membrane. Two proton pumps, namely the H^+-ATPase (V-ATPase) and the H^+-pyrophosphatase (V-PPase), located in all vacuolar membranes generate the electrochemical gradient, required to energize a large majority of the transport mediated by tonoplast proteins [Sze *et al.*, 1999, Maeshima, 2001]. Two other classes of transporters located in the vacuolar membrane mediate a directly ATP energized transport and allow the accumulation of their substrates independently from the proton motive force: cation pumps belonging to the class of the P-type ATPases and ATP-binding cassette (ABC) transporters [White & Broadley, 2003, Rea, 2007, Yazaki et al. 2009].

The ATP-binding cassette (ABC) transporters constitute a large, diverse, ubiquitous superfamily, widespread in bacteria, fungi, animal and plant kingdoms. Most of the ABC proteins are involved in the directly energized transport of a large variety of substances across various biological membranes. Compared to all other organisms, plants contain by far more ABC transporters. Most of them can be grouped in the eight major subfamilies (A-H), universally shared by living organisms [Verrier *et al.*, 2008]. In plants, ABC proteins have been shown to be implicated in diverse essential processes such as vacuolar sequestration of xenobiotics, heavy

117 metal tolerance, pigment accumulation, auxin transport, alkaloid import at
118 the plasma membrane, wax deposition on the cuticle, lipid catabolism,
119 stomatal regulation, disease/pathogen resistance or assembly of redox-
120 active cytosolic Fe/S proteins [Martinoia *et al.*, 1993, Lu *et al.*, 1998,
121 Gaedeke *et al.*, 2001, Shitan *et al.*, 2003, Pighin *et al.*, 2004, Xu & Møller,
122 2004, Lee *et al.*, 2005, Geisler & Murphy, 2006, Kim *et al.*, 2007, Rea 2007,
123 Yazaki *et al.* 2009]. However, the number of uncharacterized ABC
124 transporters remains high and their implication in plant metabolism still
125 awaits to be discovered.
126
127 Peptides are implicated in many different processes occurring in living cells.
128 In mammals they are used for example for antigene processing or as
129 hormones (Parcej and Tampe, 2010,). Furthermore, some antibiotics and
130 anticancer drugs are peptides [Dantzig *et al.*, 1992, Hori *et al.*, 1993]. In
131 bacteria, peptides can be involved in quorum sensing [Swift *et al.*, 1996]. In
132 plants, during the seed germination, peptides are first produced to supply the
133 seedling with reduced nitrogen [Higgins & Payne, 1981, Stacey *et al.*,
134 2002b]. Three gene families potentially code for peptide transporters.
135 Members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family
136 recognize di- and tripeptides, while some members of the oligopeptide
137 transport (OPT) family transport predominantly tetra- and pentapeptides,
138 though longer or shorter peptides are sometimes also translocated [Tsay *et*
139 *al.*, 2007, Rentsch *et al.*, 1995, 2007, Koh *et al.*, 2002, Stacey *et al.*, 2002a,

Waterworth and Bray, 2006, Pike *et al.*, 2009]. In mammals, three members of the ABC transporter superfamily, the so-called TAPs, have been shown to be involved in the transport of longer chain peptides. They reside either in the ER, Golgi or in lysosomal membrane and mediate the transport of peptides ranging from 6 to 59 amino acids [Kelly *et al.*, 1992, Lankat-Buttgereit and Tampé, 2003, Wolters *et al.*, 2005].

Early work on plant vacuoles has shown that vacuoles contain highly active proteases [Boller and Kende, 1979]. It was suggested that these proteases are implicated in cellular protein turnover. However, this hypothesis still awaits confirmation. Proteomic studies using Arabidopsis and barley vacuolar membranes have revealed a large number of putative vacuolar transporters with still unknown function, among also TAP homologs (Carter *et al.* 2004, Endler *et al.*, 2006, Jaquinot *et al.*, 2007). We were therefore interested to investigate whether vacuoles exhibit peptide transport activity for larger peptides of a size between 9 and 30 amino acids.

Material and Methods

Plant material. Barley (*Hordeum vulgare* L. cv. Baraka) was grown in a controlled environment chamber (16 h light (300 µE $m^{-2} \cdot s^{-1)}$ / 8 h dark cycle; 22 ºC, 60% relative humidity).

163 *Isolation of intact vacuoles.* Barely mesophyll vacuoles were isolated by a
164 slight modification described in *Rentsch and Martinoia* (1991). After
165 resuspension of the protoplasts, they were collected by centrifugation (10
166 min, 200 x g) on a cushion of osmotically stabilized Percoll (500 mM sorbitol;
167 1 mM $CaCl_2$ (Fluka Buchs, Switzerland); 20 mM MES, pH 6). Protoplasts
168 were suspended in 5 ml of a solution containing 500 mM sorbitol; 30% (v/v)
169 Percoll; 1 mM $CaCl_2$; and 20 mM MES, pH 6. This suspension was
170 overlayered with 10 ml medium A (430 mM sorbitol, 25% (v/v) Percoll, 30
171 mM K-gluconate (Fluka Buchs, Switzerland) and 20 mM HEPES-KOH pH
172 7.2) and 5 ml medium B (430 mM sorbitol, 30 mM K-gluconate and 20 mM
173 HEPES-KOH pH 7.2). After centrifugation for 10 min at 200 x g, protoplasts
174 were recovered from the upper interface, mixed with 15% (v/v) medium B;
175 and 10 mM EDTA and forced through a syringe without needle to liberate the
176 vacuoles. The protoplasts lysate was suspended in a solution containing 500
177 mM sorbitol; 20% (v/v) Percoll; and 20 mM HEPES-KOH pH 7.2. The
178 suspension was overlayered with 5 ml of medium B; 0.1% (w/v) BSA (Fluka
179 Buchs, Switzerland); and 0.2 mM DTT and 1 ml medium C (400 mM glycine-
180 betaine, 30 mM K-gluconate and 20 mM HEPES-KOH pH 7.2); 0.1% (w/v)
181 BSA; and 0.2 mM DTT. After centrifugation for 3 min at 300 rpm and 5 min
182 1200 rpm, vacuoles were recovered from the upper interface. Last
183 purification gradient was repeated with the recovered vacuoles but
184 centrifuged for 5 min at 1500 rpm. The purified vacuoles recovered from the
185 upper interface were completed with Percoll (final concentration 10% (v/v)

186 Percoll; 500 mM sorbitol; and 20 mM HEPES-KOH pH 7.2) and the
187 suspension was directly used for uptake experiments.
188
189 *Uptake experiments.* Uptake of fluorescein-labeled peptides 9-mer:
190 RRYC(ϕ)KSTEL, 18-mer: RRYQKSTELRRYC(ϕ)KSTEL, and 27-mer:
191 RRYQKSTELRRYQKSTELRRYC(ϕ)KSTEL was measured by a slight modification of
192 method described by *Martinoia* et al. (1993). For each condition and time
193 point, five polyethylene microcentrifugation tubes (400 µl capacity) were
194 prepared as follows: 70 µl of basal medium (22% Percoll pH 7.2, 430 mM
195 sorbitol, 30 mM K-gluconate, 20 mM HEPES-KOH pH 7.2, 0.1% BSA, 0.2
196 mM DTT) containing 1 mC ^3H_2O, 2 µM of one of the fluorescein-labeled
197 peptides and solutes as indicated in the figures were placed in the bottom of
198 the tubes. Experiments were started by the addition of 30 µl of the vacuole
199 suspension. Samples were rapidly overlayered with 200 µl silicone oil AR
200 200 and 60 µl medium C described in the paragraph above. Incubation was
201 interrupted by centrifugation at 10,000 x *g* for 20 s. The fluorescent peptides
202 contained in the medium C were quantified using a fluorescence plate reader
203 (λ ex/em 485/520 nm; Fusion, Packard). ^3H_2O equilibrates rapidly between
204 the medium and the vacuolar space and was used to quantify the number of
205 vacuoles by scintillation counting.
206 For competition, randomized peptide libraries were added (Uebel et al 1997)
207
208

Results

Time-dependent uptake of fluorescein-labeled peptides

In order to characterize a hypothetical vacuolar peptide transporter in plants, we isolated highly purified barley mesophyll vacuoles. Transport assays were performed using three peptides of different sizes, a 9 amino acid (aa) (RRYC(ϕ)KSTEL), a 18 aa (RRYQKSTELRRYC(ϕ)KSTEL) and a 27 aa (RRYQKSTELRRYQKSTELRRYC(ϕ)KSTEL) peptide, where ϕ symbolizes the fluorescein fluorophore. Uptake experiments were performed at a peptide concentration of 2 µM in the presence or absence of 4 mM MgATP. Uptake of all three peptides was strictly MgATP-dependent (Fig. 1A, B and C). In the absence of MgATP, no uptake could be observed, while in the presence of MgATP, efficient peptide uptake occurred which was linear for at least 20 minutes. The ATP-dependent transport rates increased with the size of the peptide. They were 0.59±0.14, 0.88±0.13 and 1.78±0.50 pmol $ml^{-1} \cdot min^{-1}$ for the 9, 18 and 27 amino acid long peptide, respectively.

Energization of the peptide transporter

Vacuoles have an acidic pH; therefore, the ATP-dependent peptide transport into barley vacuoles might occur by a secondary energized mechanism utilizing the proton motive force or directly by MgATP-hydrolysis by ABC-type transporters. To distinguish between these two mechanisms, we performed transport experiments using different inhibitors. Since uptake was

232 linear for at least 20 min, peptide uptake rates were determined by
233 subtracting the 2 min from the 20 min value. This allowed correcting for
234 unspecific adsorption. Compared to MgATP, MgGTP, which is known to be
235 able to partially substitute for MgATP in the case of ABC transporters, was
236 able to catalyze the peptide transport to 30 to 50% (Fig. 2). Interestingly,
237 MgGTP was more efficient in energizing the transport of the longer peptides.
238 The non-hydrolysable ATP analogue, AMP-PMP failed to catalyze peptide
239 transport, indicating that energy is required to drive the transport of the
240 peptides. Valinomycin, a K^+ specific ionophore, which dissipates the
241 membrane potential, and bafilomycin, a highly specific inhibitor of the
242 vacuolar H^+-ATPase, did not inhibit the transport of the different peptides.
243 Interestingly, valinomycin had even a promoting effect in case of the 27-mer.
244 Vanadate, is an efficient inhibitor for most ABC-type protein-mediated
245 transport processes, also inhibited the ATP-driven peptide transport. This
246 inhibition was more pronounced (80%) for the shorter peptides, while
247 transport of the 27 aa peptide was only inhibited by 50% (Fig. 2).
248 Since the inhibitory effect of vanadate at a concentration of 0.5 mM was
249 different for short and long peptides, we wondered whether the
250 concentration-dependence of the vanadate inhibition was different for the
251 nine and twenty-seven amino acid long peptides. Raising the vanadate
252 concentration did not consistently lead to a higher inhibition than observed at
253 0.5 mM (Fig. 3). Furthermore, the concentration required to inhibit the
254 transport rate to 50% of the maximal vanadate inhibition, were 75 µM and 80

255 μM, for the 9 aa and 27 aa peptide, respectively. Similar IC_{50} values have
256 been already reported for other ABC mediated transport processes [Wolters
257 *et al.*, 2005].
258 We determined also the concentration dependency for MgATP using the 9
259 aa peptide. The data clearly fitted by the Michaelis-Menten equation with an
260 apparent K_m^{MgATP} value of 146.6 ± 93.7 μM (Fig. 4), a value which is similar to
261 affinity reported for other ABC transporters [Rea *et al.,* 1998].
262
263 **Concentration-dependency of the peptide transporter and impact of the**
264 **peptide length**
265 As shown in Fig. 5, the transport rates for the 9 aa peptide followed a
266 Michaelis-Menten kinetic. As this experiment required a large amount of
267 peptide, increasing the concentration until reaching saturation was not
268 possible. From the data available, an apparent K_m^{pep} of about 15 μM and a
269 maximal transport activity (V_{max}) of about 8 pmol $\mu l^{-1} \cdot min^{-1}$ were determined.

270 Furthermore, we were interested to investigate how the transport of the 9 aa
271 peptide was affected by peptides of different length. To this end, we
272 performed uptake experiments using 2 μM of the fluorescent 9 aa peptide in
273 the presence of 20 μM of unlabeled randomized peptide libraries of
274 increasing size (X_i, i = 4, 7, 10, 11, 17, 23, 35 and 53 residues, Fig. 6, see
275 Materials and Methods). The advantage of these randomized peptide
276 libraries is that they do not relay on a single sequence. The transport activity
277 of the labeled peptide showed no reduction in the presence of a small

peptide of 4 amino acid residues. The peptide with 7 aa exhibited a 20% inhibition, while the peptide with 10 and more aa inhibited uptake of the 9 aa peptide by about 50%. This value can be expected taking into account a Km of 15 to 20 µM. Longer peptides had the tendency to be slightly more inhibitory. However, this inhibition study suggests that while the transport activity increases with the size of the peptide the affinity of the transporter for the longer chain peptides remains the same.

Discussion

Peptides play an important role not only in nitrogen nutrition but also in various processes occurring within living cells [Higgins and Payne, 1981, Stacey *et al.*, 2002b]. Despite the identification of a large number of putative peptide transporters in the *Arabidopsis* genome, little is known about their activity, localization and involvement in the whole plant metabolism. All plant peptide transporters described so far transport small peptides up to 5 amino acids. Only AtOPT6 was shown to transport also peptide as long as 13 amino acids [Pike *et al.*, 2009]. Large peptides may be generated by endoproteases as well by the proteasome complex. The universal ubiquitin-proteasome complex pathway is the best-defined protein degradation pathway [Smalle & Viestra, 2004]. The proteasome complex exhibits three proteolytic activities, a chymotrypsin-like, a trypsin-like, and a peptidyl-

glutamyl peptide-hydrolyzing activity. Combined together, these proteases generate peptides of a size between 3 and 30 amino acids. The universal ubiquitin-proteasome complex pathway together with the less-well characterized cytoplasmic and plastid degradation pathways produce the major part of the peptides found in the plant cell [Sakamoto, 2006]. So far, the subsequent step of this protein turnover process, *i.e.* the final degradation of the resulting peptides leading to the production of amino acids, which can be reused for *de novo* protein synthesis, is less understood. Since a long time, the presence of hydrolytic enzymes in vacuoles had been demonstrated [Boller and Kende, 1979]. It was however not clear, whether peptides have to be imported into the vacuole for their final degradation.

In contrast to plants, transporters for long chain peptides have been well characterized in animals. These transporters belong to the so-called TAP (Transporter associated with Antigenic Processing) family. In humans, TAP1 (ABCB2) and TAP2 (ABCB3) form a heterodimer which transports peptides generated by the proteasome complex into the ER where they are loaded onto a newly synthesized MHC class I complex. The MHC I-peptide complex is exported to the cell surface to be exposed to T-cells. By means of its function, this heterodimer was shown to play a central role in the immune system. (Trowsdale *et al.*, 1990, Bahram *et al.*, 1991, Higgins *et al.*, 1992, Abele and Tampé, 2004). . In mammals the third member of this family is the so-called TAP-like, which forms homodimers and is a peptide transporter

323 located in the lysosomal membrane [Wolters *et al.*, 2005; Demirel *et al.*,
324 2007].
325 The finding of TAP-like transporters in the plant vacuole by different
326 proteomic approaches encouraged us to investigate the vacuolar transport
327 activity for larger peptides [Endler *et al.*, 2006]. We chose peptides, which
328 had a similar amino acid sequences as used to characterize the human TAP
329 heterodimer mediating peptide transport [Lankat-Buttgereit and Tampé,
330 2003]. All three fluorescein-labeled peptides tested were transported across
331 the tonoplast in a strictly ATP-dependent way. As expected for an ABC-type
332 catalyzed transport, GTP but not AMP-PMP could substitute for ATP.
333 Furthermore, peptide transport was inhibited by vanadate, but not by
334 bafilomycin or valinomycin. Vanadate inhibition was more pronounced for
335 shorter peptides, whereas the transport of the 27-aa peptide was inhibited
336 only about 50%. Valinomycin increased the transport activities in the case of
337 larger peptides (Fig. 2). This stimulating effect of valinomycin could be
338 related to the excess of positive charges of the peptide and the fact that the
339 longer peptides used in this study contained an increased number of positive
340 charges. Destroying the trans-vacuolar membrane potential may therefore
341 reduce the energy for the transfer of the peptides and this would be most
342 pronounced for the 27 aa peptide, since it contains six net positive charges.
343
344 Our data suggests that the vacuole has at least one ABC-type protein that
345 transports peptides from the cytosol into the vacuole. The transporter

346 exhibited an apparent affinity of approximate 15 μM for 9 aa peptides, which
347 is similar to that of the human ABCB9/TAPL (6.8 μM) [Wolters *et al.*, 2005].
348 This high affinity of the transporter would indicate an efficient transport of the
349 peptides from the cytosol into the vacuole. It is likely that the peptides
350 transported into the vacuole are subsequently degraded to free amino acids
351 by vacuolar endoproteases and exopeptidases. We therefore hypothesize
352 that the proteasome and maybe other, so far unknown cytosolic proteolytic
353 activities produce peptides, which are readily transported into the vacuole in
354 order to be degraded and recycled to sustain plant metabolism. Whether
355 peptides generated by plastidic proteases are exported into the cytoplasm
356 and finally transported into the vacuole is so far unknown. TAP-like plant
357 transporters have been localized in the vacuolar membrane using a GFP
358 fusion protein as well as by proteomic approaches [Yamaguchi *et al.*, 2002,
359 Endler *et al.*, 2006]. Further studies will show whether these TAP-like
360 proteins act as long-chain peptide transporters also in plants. Arabidopsis
361 AtTAP2/AtABCB27/ASL1 and the barley TAP, HvID17, have been shown to
362 be related with other processes. HvID17 is strongly induced by iron
363 deficiency and has been proposed to be implicated in iron homeostasis
364 [Yamaguchi *et al.*, 2002]. AtTAP2/AtABCB27, which was also found to be
365 localized in the vacuolar membrane in a proteome study and by using a GFP
366 tagged construct, has been proposed to be involved in aluminium tolerance,
367 since the corresponding knock-out mutant exhibited reduced root growth
368 when grown on Al^{3+} containing medium [Jaquinot *et al.*, 2007, Larsen *et al.*,

2007]. Further studies have to be performed to identify which transporter is responsible for the long-chain peptide transport and which role it plays in nitrogen metabolism of the plant.

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References

Abele R, Tampé R. 2004. The ABCs of immunology: structure and function of TAP, the transporter associated with antigen processing. *Physiology* **19**, 216-224.

Bahram S, Arnold D, Bresnahan M, Strominger JL, Spies T. 1991. Two putative subunits of a peptide pump encoded in the human major histocompatibility complex class II region. *Proceedings of the National Academy of Sciences, USA* **88**. 10094 -10098.

Boller T, Kende H. 1979. Hydrolytic enzymes in central vacuole of plant cells. *Plant Physiology*, **63**, 1123-1132.

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV. 2004. The vegetative vacuole proteome of Arabidopsis thaliana reveals predicted and unpredicted proteins. *The Plant Cell* **16**: 3285-3303.

Dantzig AH, Tabas LB, Bergin L. 1992. Cefaclor uptake by the protondependent dipeptide transport carrier of human intestinal Caco-2 cells and comparison to cephalexin uptake. *Biochimica et Biophysica Acta* **1112**, 167–173.

Demirel Ö, Waibler Z, Kalinke U, Grünebach F, Appel S, Brossart P, Hasilik A, Tampé R, Abele R. 2007. Identification of a lysosomal peptide transport system induced during dendritic cell development. *The Journal of Biological Chemistry* **282**, 37836-37843.

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginski S, Martinoia E, Schmidt UG. 2006. Identification of a vacuolar sucrose transporter in barley and Arabidopsis mesophyll cells by a tonoplast proteomic approach. *Plant Physiology* **141**, 196-207.

Gaedeke N, Klein M, Kolukisaoglu U, Forestier C, Muller A, Ansorge M, Becker D, Mamnun Y, Kuchler K, Schulz B, Mueller-Roeber B, Martinoia E. 2001.The *Arabidopsis thaliana* ABC transporter AtMRP5 controls root development and stomatal movement. *The EMBO Journal* **20**, 1875-1887.

Geisler M, Murphy AS. 2006. The ABC of auxin transport: the role of the P-glycoproteins in plant development. *The FEBS Journal* **580**, 1094-1102.

Higgins CF. 1992**.** ABC TRANSPORTERS: from microorganisms to man. *Annual Review of Cell Biology*. **8**, 67-113.

Higgins CF, Payne JW. 1981. The peptide pools of germinating barley grains: relation to hydrolysis and transport of storage proteins. *Plant Physiology* **67**, 785-792.

Hori R, Tomita Y, Katsura T, Yasuhara M, Inui KI, Takano M. 1993. Transport of bestatin in rat renal brush-border membrane vesicles. *Biochemical Pharmacology* **45**, 1763–1768.

Hörtensteiner S, Feller U. 2002. Nitrogen metabolism and remobilization during senescence. *Journal of Experimental Botany* **53**, 927-937.

Jaquinod M, Villiers F, Kieffer-Jaquinod S, Hugouvieux V, Bruley C, Garin J, Bourguignon J. 2007.A proteomics dissection of *Arabidopsis thaliana* vacuoles isolated from cell culture. *Molecular Cell Proteomics* **6**, 394-412.

Kelly AP, Powis SH, Kerr LA, Mockridge I, Elliot T, Bastin J, Uchanska-Ziegeler A, Trowsdale J, Townsend A. 1992. Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. *Nature* **355**, 641-644.

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y. 2007. The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *The Plant Journal* **50**, 207-218.

Koh S, Wiles AM, Sharp JS, Naider FR, Becker JM, Stacey G. 2002. An oligopeptide transporter gene family in Arabidopsis. *Plant Physiology* **128**, 21-29.

Lankat-Buttgereit B, Tampé R. 2003. The transporter associated with antigen processing (TAP) a peptide transport and loading complex essential for cellular immune response. *ABC Proteins : From Bacteria to Man* ch **26**, 533-550, Elsevier Science Ltd.

Larsen PB, Cancel J, Rounds M, Ochoa V. 2007**.** Arabidopsis *ASL1* encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminium toxic environment. *Planta* **225**, 1447-1458.

Lee M, Lee K, Lee J, Noh EW, Lee Y. 2005. AtPDR12 contributes to lead resistance in Arabidopsis. *Plant Physiology* **138**, 827-836.

Lu YP, Li ZS, Drozdowicz YM, Hörtensteiner S, Martinoia E, Rea PA. 1998. AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites : functional comparisons with AtMRP1. *The Plant Cell* **10**, 267-282.

Maeshima M. 2001. Tonoplast transporters: organization and function. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 469-497.

Martinoia E, Grill E, Tommasini R, Kreuz K, Amrhein N. 1993. ATP-dependent S-conjugate 'export' pump in the vacuolar membrane of plant. *Nature* **364**, 247-249.

Martinoia E, Maeshima M, Neuhaus EH. 2007. Vacuolar tranporters and their essential role in plant metabolism. *Journal of Experimental Botany* **58**, 83-102.

Parcej D, Tampé R. 2007. Caught in the act: an ABC transporter on the move. *Structure* **15**, 1028-1030.

Pighin JA, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst, L, Samuels AL. 2004. Plant cuticular lipid export requires an ABC transporter. *Science* **306**, 702-704.

Pike S, Patel A, Stacey G, Gassmann W. 2009. Arabidopsis OPT6 is an oligopeptide transporter with exceptionally broad substrate specificity. *Plant Cell Physiology* **50**, 1923-1932.

Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E. 1998. From vacuolar GS-X pumps to multispecific ABC transporters. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 727-760.

Rea PA. 2007. Plant ATP-binding cassette transporters. *Annual Review of Plant Physiology and Plant Molecular Biology* **58**, 347-375.

Rentsch D, Martinoia E. 1991**.** Citrate transport into barley mesophyll vacuoles - comparison with malate-uptake activity. *Planta* **184**, 532-537.

Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S, Frommer WB. 1995. NTR1 encodes a high affinity oligopeptide transporter in Arabidopsis. *FEBS Letters* **370**, 264-268.

Sakamoto W. 2006. Protein degradation machineries in plastids. *Annual Review of Plant Biology*. **57**, 599-621.

Shitan N, Bazin I, Dan K, Obata K, Kigawam K, Ueda K, Sato F, Forestier C, Yazaki K. 2003. Involvement of CjMDR1, plant multidrug resistance-type ATP-binding cassette protein, in alkaloid transport in *Copis japonica*. *Proceedings of the National Academy of Sciences, USA* **100**, 751-756.

Smalle J, Vierstra RD. 2004**.** The ubiquitin 26S proteasome proteolytic pathway. *Annual Review of Plant Biology*, **55**, 555-590.

Stacey MG, Koh S, Granger C, Becker JM. 2002a. Peptide transport in plants. *Trends in Plant Science* **7**, 257-263.

Stacey MG, Koh S, Becker JM, Stacey G. 2002b. AtOPT3, a member of the oligopeptide transporter family, is essential for embryo development in Arabidopsis. *The Plant Cell* **14**, 2799-2811.

Swift S, Throup JP, Williams P, Salmond GPC, Stewart GSAB. 1996. Quorum sensing: A population density component in the determination of bacterial phenotype. *Trends in Biochemical Sciences* **21**, 214–219.

Sze H, Li X, Palmgren MG. 1999. Energization of plant cell membranes by H^+-pumping ATPases:regulation and biosynthesis. *The Plant Cell* **11**, 677-689.

Tsay YF, Schroeder JI, Feldman KA, Crawford NM. 1993. The herbicide sensitivity gene *CHL1* of Arabidopsis encodes a nitrate-inducible nitrate transporter. *Cell* **72**, 705-713.

Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A. 1990. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. *Nature* **348**, 741-744.

Uebel, S., Kraas, W., Kienle, S, Wiesmuller, K.H., Jung, G., Tampe, R. 1997 Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proceedings of the National Academy of Sciences, USA* **88**. 10094 -10098.

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki Waterworth WM, Bray CM. 2006**.** Enigma variations for peptides and their transporters in higher plants. *Annals of Botany* **98**, 1-8.
White PJ, Broadley MR. 2003. Calcium in plants. *Annals of Botany* **92**, 487-511.

Wolters JC, Abele R, Tampé R. 2005. Selective and ATP-dependent translocation of peptides by the homodimeric ATP binding cassette transporter TAP-like (ABCB9). *The Journal of Biological Chemistry* **280**, 23631-23636.

Xu XM, Møller SG. 2004. AtNAP7 is a plastidic SufC-like ATP-binding cassette/ATPase essential for Arabidopsis embryogenesis. *Proceedings of the National Academy of Sciences, USA* **101**, 9143-9148.

Yamaguchi H, Nishizawa NK, Nakanishi H, Mori S. 2002. IDI7, a new barley iron-regulated ABC transporter from barley roots, localizes to the tonoplast. *Journal of Experimental Botany* **53**, 727-735.

Yazaki K, Shitan N, Sugiyama A, Takanashi K. 2009**.** Cell and molecular biology of ATP-binding cassette proteins in plants. *International Review of Cell and Molecular Biology* **276**, 263-99.

Figure legends

Fig. 1: Time-dependent uptake of peptides into isolated barley mesophyll vacuoles. Vacuoles were incubated with 2 µM of three different fluorescein(ϕ)-labeled peptides; 9-mer: RRYC(ϕ)KSTEL (A), 18-mer: RRYQKSTELRRYC(ϕ)KSTEL (B) and 27-mer: RRYQKSTEL-RRYQKSTELRRYC(ϕ)KSTEL (C) in the absence (△) or presence (▲) of 4 mM MgATP. Peptide transport was quantified by fluorescence detection as described in Materials and Methods. At the time points indicated, incubation

was interrupted by removing vacuoles from the incubation medium via centrifugation through silicon oil. Each time point is the mean of 5 replicates, and error bars represent S_D.

Fig. 2: Effect of nucleotides and inhibitors on peptide uptake. Comparison of the transport activities of labeled 9-mer (A), 18-mer (B) and 27-mer (C) peptides in response to nucleotides (MgATP, MgGTP and AMP-PNP, 4 mM) and inhibitors (0.5 mM vanadate, 10 µM valinomycin and 0.1 µM bafilomycin). Peptide transport was quantified by fluorescence detection and transport activities were related to transport in the presence of MgATP (100%). Values are means of two to three independent experiments each with 5 replicates, error bars represent S_D.

Fig. 3: Inhibition of 9- and 27-mer peptide uptake as a function of ortho-vanadate concentration. Uptake of fluorescein-labeled 9-mer (■) and 27-mer (▲) peptides into barley mesophyll vacuoles was determined in the presence of increasing ortho-vanadate concentrations. Transport was carried out in the presence of 4 mM MgATP.

Fig 4. MgATP concentration dependent uptake of the 9-mer peptide

ATP dependency of the fluorescein-labeled 9-mer (■) transport activity was established by increasing MgATP concentrations. Barley mesophyll vacuoles were incubated for 20 min in the presence of 2 µM 9-mer peptide. MgATP concentrations were kept constant by the addition of phosphocreatine (10

mM) and phosphocreatine kinase (16 U • ml^{-1}). Peptide transport was quantified by fluorescence detection and inhibition of the transport activity was related to transport in the absence of vanadate (100%). Results are means of two experiments per peptide. Each concentration is the mean of two independent experiments, each with 5 replicates and error bars represent S_D.

Fig. 5. Concentration-dependent uptake of the 9-mer peptide in barley mesophyll vacuoles.

Vacuoles were incubate for 20 min in the presence of 4 mM MgATP and fluorescein-labeled peptide (RRYC(Φ) KSTEL). Concentration dependence of the peptide transport activity was determined by increasing peptide concentrations. The data were fitted by the Michaelis-Menten equation. Peptide transport was quantified by fluorescence detection. Each concentration is the mean of 5 replicates and error bars represent S_D.

.**Fig. 6. Inhibition of the uptake of the 9-mer peptide by peptides of increasing size.** Transport activities were determined by incubating barley mesophyll vacuoles in the presence of fluorescein-labeled 9-mer peptide (RRYC(Φ)KSTEL), 4 mM MgATP and one of the non-labeled randomized peptide libraries of different length (X_i, i = 4,7,10, 11, 17, 23, 35 and 53 residues, see Materials and Methods). The concentration of labeled peptide was 2 µM, non-labeled peptides were added at a 10 fold excess (20 µM) and

incubation time was 20 min. Peptide transport was quantified by fluorescence detection and transport rates were related to transport in the absence of an unlabeled peptide (100%). The data represent means of two to three independent experiments each with 5 replicates for each X_l-mer peptide, error bars represent S_D.

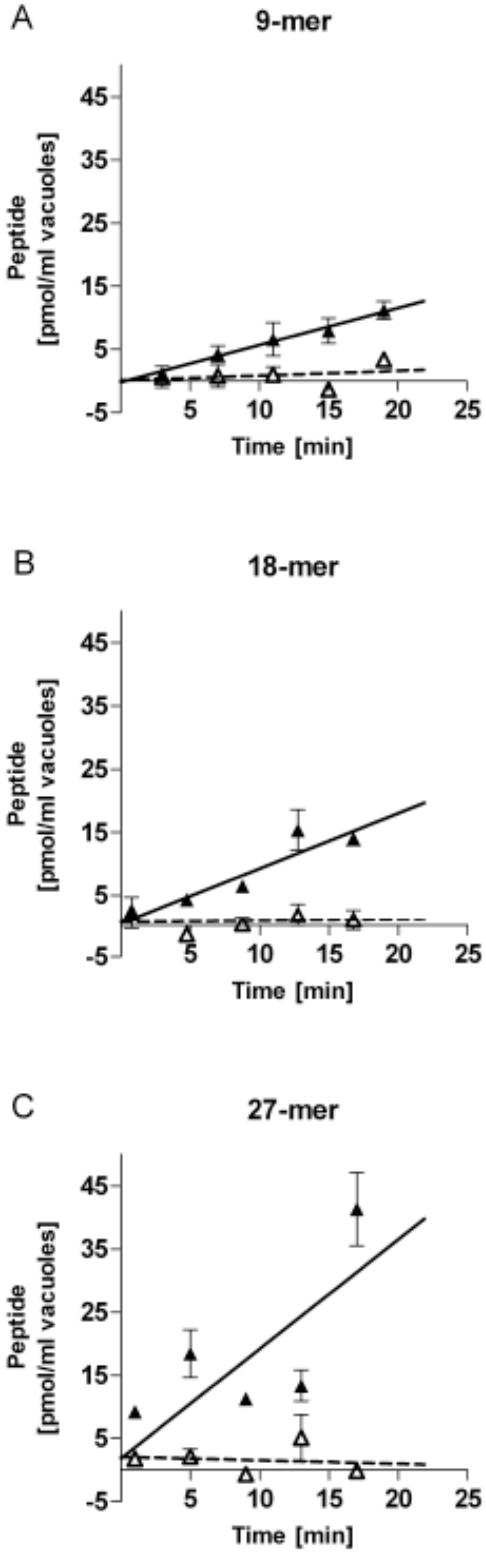
A
9-mer
Peptide
[pmol/ml vacuoles]
45
35
25
15
5
-5
5
10
15
20
25
Time [min]
B
18-mer
Peptide
[pmol/ml vacuoles]
45
35
25
15
5
-5
5
10
15
20
25
Time [min]
C
27-mer
Peptide
[pmol/ml vacuoles]
45
35
25
15
5
-5
5
10
15
20
25
Time [min]

Figure 1

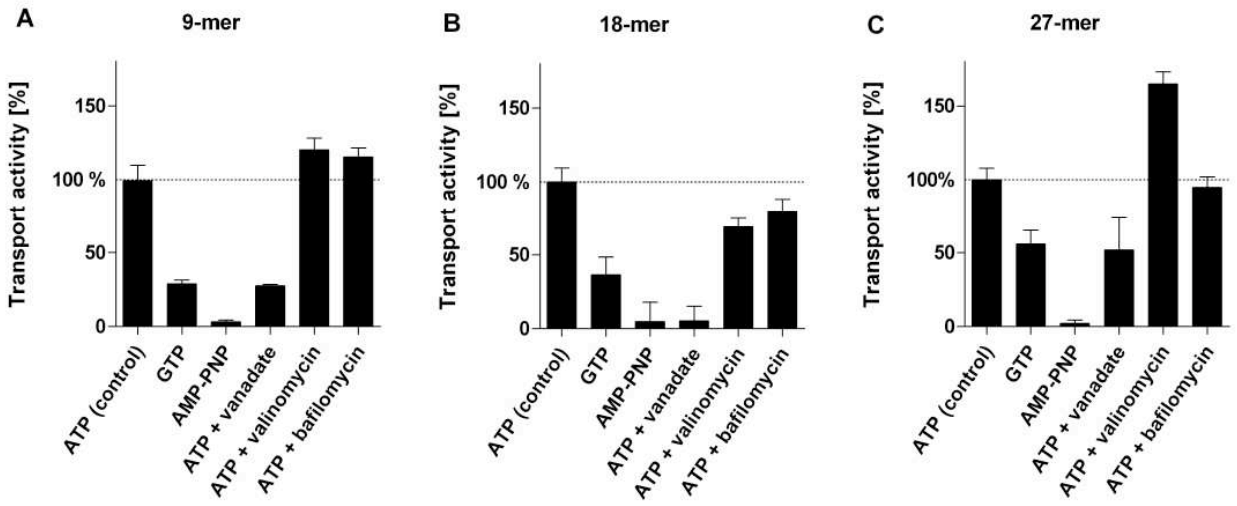
A
9-mer
B
18-mer
C
27-mer
Transport activity [%]
150
100 %
100%
50
0
ATP (control)
GTP
AMP-PNP
ATP + vanadate
ATP + valinomycin
ATP + bafilomycin

Figure 2

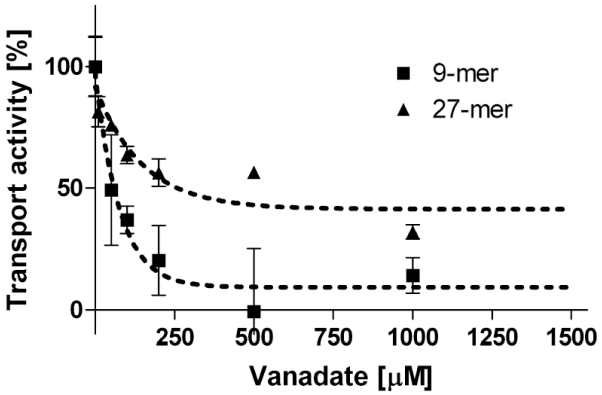
Transport activity [%]
100
50
0
250
500
750
1000
1250
1500
Vanadate [μM]
9-mer
27-mer

Figure 3

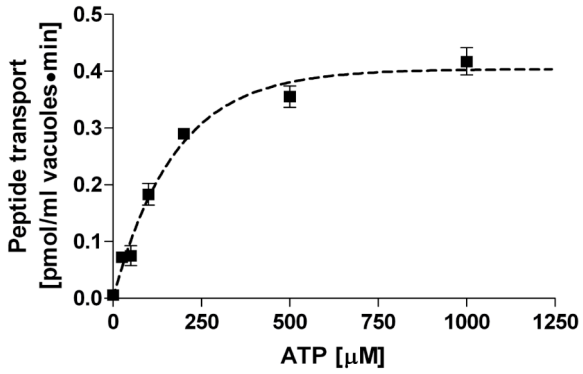
Peptide transport
[pmol/ml vacuoles•min]
0.5
0.4
0.3
0.2
0.1
0.0
0
250
500
750
1000
1250
ATP [μM]

Figure 4

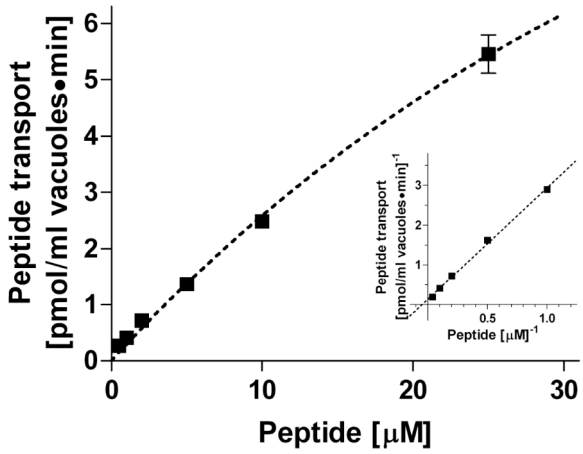
Peptide transport
[pmol/ml vacuoles•min]
Peptide [μM]
0
10
20
30
1
2
3
4
5
6
Peptide transport
[pmol/ml vacuoles•min]-1
Peptide [μM]-1
0.5
1.0

Figure 5

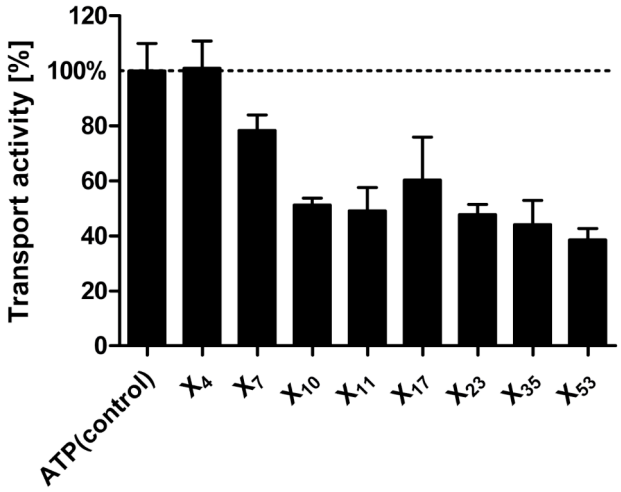
Transport activity [%]
120
100%
80
60
40
20
0
ATP(control)
X4
X7
X10
X11
X17
X23
X35
X53

Figure 6